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Continuous Culture of Aspergillus phoenicis *QM 329 for the Production of Cellobiase*

INTRODUCTION

Although the cellulase system of *Trichoderma reesei* contains all the enzymes necessary for complete hydrolysis of cellulose, the levels of cellobiase (β-glucosidase) are suboptimal for saccharification purposes.[1] Increasing the level of cellobiase enhances the rate of saccharification of cellulose by reducing the level of cellobiose, an inhibitor[2,3] of the cellulase system. Even then, cellulase requirements are high,[4] so fermentation studies with *T. reesei* have concentrated in optimization of cellulase productivity and it is probable that large increments in cellobiase levels can not be attained for this organism without sacrificing cellulase productivity.[5] Optimization of the β-glucosidase level can be achieved through supplementation of this enzyme from a separate fermentation. This communication discusses the production of β-glucosidase from *Aspergillus phoenicis* in continuous culture.

MATERIALS AND PROCEDURES

For this study, *Aspergillus phoenicis* QM329 was used. Slants of the stock cultures were maintained on potato dextrose agar at 24°C. The salt medium used was that described by Mandels and Reese[6] except that urea was not added. Tween 80 was added at a 0.05% level. Proteose peptone was not used. Antifoam SAG-100 (Union Carbide Corp., New York, NY) was added for foam control. A 2-L fermentor was used (BioFlo from New Brunswick Scientific, New Brunswick, NJ) with a working volume of between 1300 and 1500 mL. Nutrients were fed in at a constant rate, but the harvest was intermittent to minimize internal recycle of biomass. The fermentor culture was spore-inoculated into medium containing the salts and 1% glucose. The feed, containing salts and 1% corn dextrin (Fischer Scientific, Fairlawn, NJ), was started generally 24 h after inoculation, when the glucose was consumed. The pH was controlled at 3.0 with ammonia addition. Based on preliminary shake-flask data, the optimum growth temperature of 35°C was used. To minimize wall growth, the fermentation was stopped after 3–5 vol of feed medium were consumed at one dilution rate. Samples were taken at intervals corresponding to the fermentor residence time (i.e., at a dilution of 0.04 h^{-1}, one sample every 25 h). A steady state was assumed to be reached when the following occurred: 1) the carbon dioxide level in the exhaust gas remained constant (carbon dioxide measured with an infrared analyzer, Mine Safety Products Co., Pittsburgh, PA), 2) the dry weight of the fermentor biomass remained constant between two samples; and 3) the whole-culture enzyme levels were the same in both the fermentor and harvest vessel. The harvest vessel was sampled and emptied after harvesting 1 fermentor vol. Assays for mycelial dry weight and cellobiase levels are described elsewhere.[7]

RESULTS AND DISCUSSION

Although *Aspergillus phoenicis* will produce cellobiase (β-glucosidase) from glucose without an exogenously supplied inducer, use of certain carbon sources results in higher levels of β-glucosidase.[8] For the economical production of β-glucosidase from *A. phoenicis*, cornstarch would be the preferred carbon source. It is both relatively inexpensive and good for β-glucosidase production. For this study in continuous culture, however, corn dextrin was used because of its greater solubility, making it an easier substrate with which to work.

Figure 1 shows the total mycelial-bound and cell-free β-glucosidase levels at various dilution

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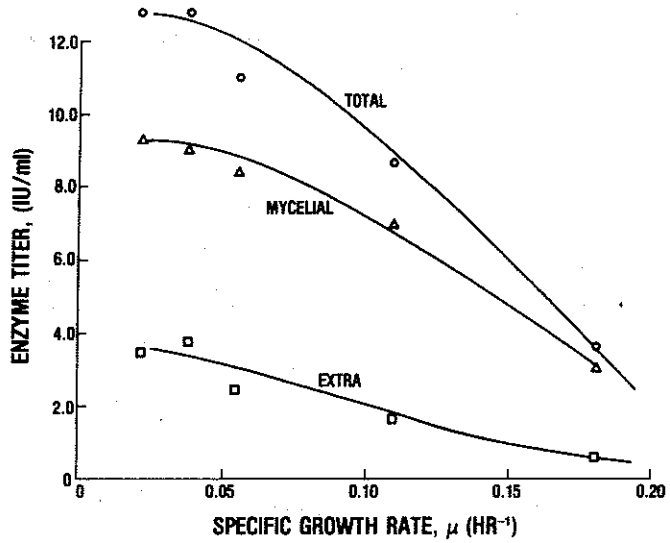

Fig. 1. Production of β-glucosidase in continuous culture with 1% corn dextrin. (EXTRA) (□) extramycelial activity, (MYCELIAL) (△) mycelial-associated activity, (TOTAL) (○) mycelial plus extramycelial.

rates. At low dilution rates (below 0.05 h^{-1}), the enzyme was approximately 70% mycelial-associated and 30% cell-free. The enzyme yield was approximately 1.3 IU/mg substrate which is slightly higher than what would be expected from a simple batch fermentation[8] for a 1% carbohydrate carbon source. The total enzyme titer decreased with increasing dilution rate and the percent of enzyme associated with the mycelium increased (Fig. 1). The decrease in enzyme titer with increasing dilution rate can be explained in part by the rising level of residual glucose (Fig. 2). The enzyme level would also fall if its production were not linked to growth.[9]

Volumetric enzyme productivity at various dilution rates (D) is depicted in Figure 3. Since the dry weight (ca. 4.5 g/L) varied only slightly over these dilution rates, specific enzyme productivity (IU g^{-1} biomass h^{-1}) follows the same pattern. For a product strictly linked to growth, productivity should be linear with growth rate until the critical dilution is approached and should also have its intercept at the origin.[9] For dilutions below 0.05 h^{-1} (Fig. 3), the β-glucosidase production appears to be linked to growth. The curve is not linear above $D = 0.05\ h^{-1}$. Maxi-

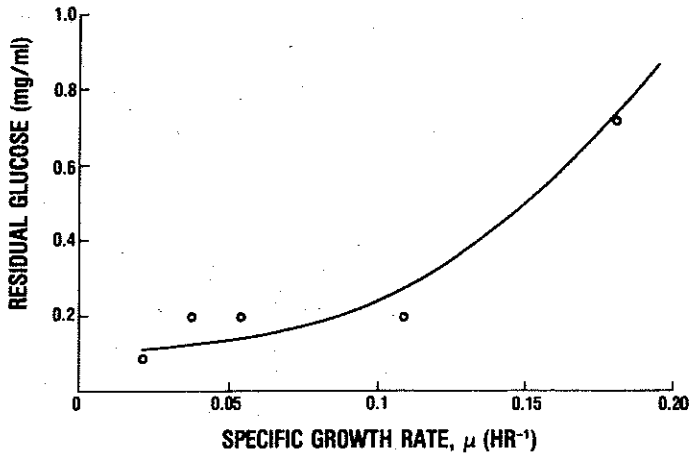

Fig. 2. Effect of growth rate on residual glucose in continuous culture of *A. phoenicis* with 1% corn dextrin.

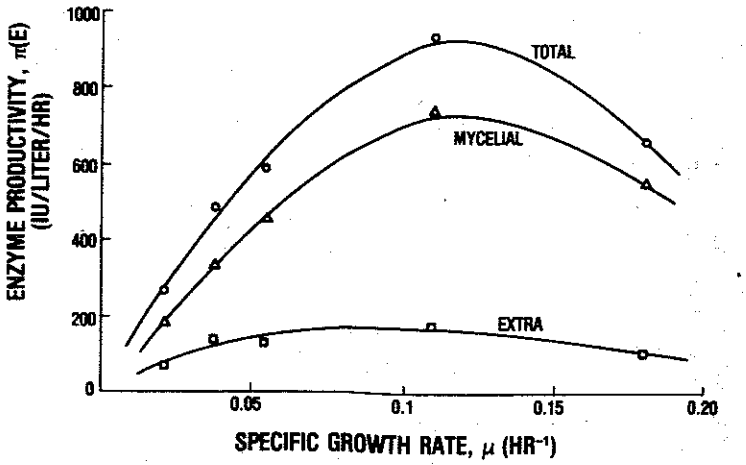

Fig. 3. Effect of growth rate on production of β-glucosidase in continuous culture with 1% corn dextrin. Symbols are the same as in Fig. 1.

mum productivity of 211 IU g^{-1} h^{-1} (950 IU L^{-1} h^{-1}) occurs at a dilution ca. 0.11 h^{-1}. Productivity declines above this dilution as the critical dilution is reached. Table I compares β-glucosidase production in continuous culture with simple batch fermentation.

Measurement of respiratory activity at the various growth rates allows the calculation of both yield and maintenance parameters for this fungus. The specific rate of carbon dioxide evolution versus the specific growth rate is linear over the range of growth rates studied (Fig. 4). The slope of the line is the inverse of the true yield and the intercept represents the maintenance coefficient.[10] From a least-squares analysis, the true yield ($Y_{x/O}$) was found to be 0.074 g biomass/mmol
oxygen and the maintenance was 0.35 mmol oxygen g^{-1} biomass h^{-1} (assuming a respiratory coefficient of 1).

Biomass productivity (Fig. 5) was fairly linear with the specific growth rate for dilutions below 0.10 h^{-1}. At $D = 0.18$ h^{-1}, a decrease in the biomass concentration with a coincident increase in background glucose indicated that the critical dilution rate was being approached.

To calculate the critical dilution rate, the fermentation was run at $D = 0.74$ h^{-1}. The slope of the straight line resulting from a semilog plot (Fig. 6) of decreasing dry weight versus time gave the quantity $\mu_{max} - D$, where μ_{max} represents the maximum specific growth rate. A least-squares analysis of the data showed the slope to be -0.46 h^{-1}. The maximum specific growth rate (0.74 − 0.46) was calculated to be 0.28 h^{-1}.

TABLE I
Comparison of β-Glucosidase Production in Continuous Culture with Simple Batch

Parameter	Continuous	Batch[a]
Maximum enzyme titer	13.0 IU/mL	10.0 IU/mL
Yield (*E/S*)	1.3 IU/mg	1.0 IU/mg
Maximum specific productivity	211 IU g^{-1} h^{-1}	46 IU g^{-1} h^{-1}
Maximum volumetric productivity	950 IU L^{-1} H^{-1}	229 IU L^{-1} h^{-1}

[a]From Allen and Sternberg, ref. 8.

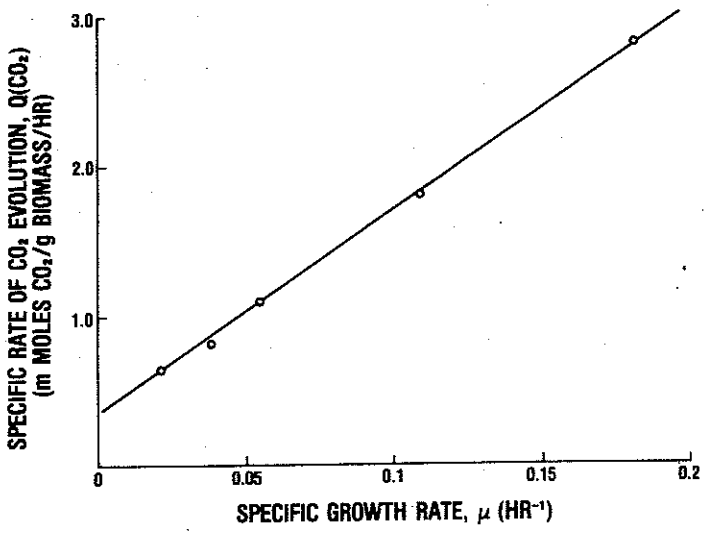

Fig. 4 Carbon dioxide evolution in continuous culture of *A. phoenicis* on 1% corn dextrin.

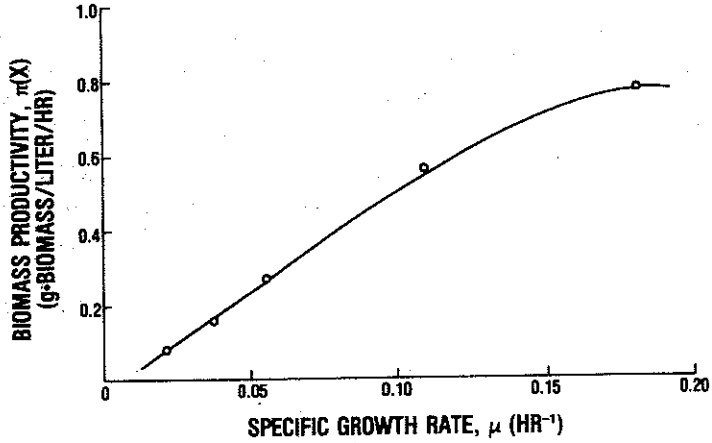

Fig. 5. Production of biomass in continuous culture of *A. phoenicis* on 1% corn dextrin.

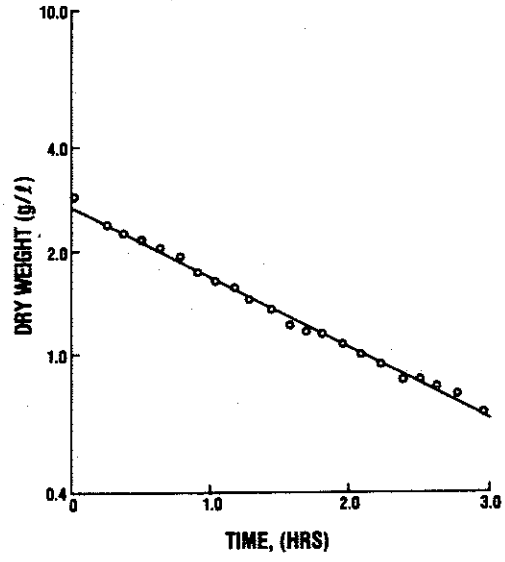

Fig. 6. Decrease in biomass of *A. phoenicis* at $D = 0.74\ h^{-1}$.

CONCLUSIONS

Continuous culture of *A. phoenicis* is attractive for the production of β-glucosidase. It has been shown that for the *in vitro* saccharification of cellulose with *Trichoderma reesei* cellulase, 50% of the cellulase can be replaced with equivalent units of β-glucosidase with no loss in rate of saccharification.[1,4] Since the maximum productivity of cellulase (*T. reesei*) is ca. 180 IU L^{-1} h^{-1},[11] and that of β-glucosidase is 950 IU L^{-1} h^{-1}, the total enzyme cost for cellulose hydrolysis can be reduced significantly.

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